

AD _____

Award Number:
W81XWH-13-1-0121

TITLE:
Understanding the Delay in Onset of Paget's Disease of Bone

PRINCIPAL INVESTIGATOR:
Marc F. Hansen, Ph.D.

CONTRACTING ORGANIZATION:
University of Connecticut
Farmington, CT 06032-1956

REPORT DATE:
September 2014

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT:

☒ Approved for public release; distribution unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE September 2014		2. REPORT TYPE Annual		3. DATES COVERED 30 Sep 2013 - 29 Aug 2014	
4. TITLE AND SUBTITLE Understanding the Delay in Onset of Paget's Disease of Bone				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-13-1-0121	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Marc F. Hansen, Ph.D. E-Mail: mhansen@uchc.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Connecticut 263 Farmington Avenue Farmington, CT 06032-1956				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT One of the key questions in Paget's disease of bone (PDB) is the nature of the "trigger" for initiation of the disease. Inheritance of a predisposing mutation and childhood infection with Measles virus are important, but apparently insufficient to initiate the disease until a significant period of time has passed. Understanding what occurs in this intervening time period is the goal of this proposal. Our model involves the opposing action of two Measles virus genes. The MVV gene suppresses gene expression as part of the virus' ability to promote persistent infection. The MVNP gene appears to activate gene expression to promote acute infection. In our model, when Measles virus infects a bone cell, the MVV gene creates a latent infection by suppressing gene expression in the cell. Then, a chance genetic event results in loss of the MVV gene and unmasking the MVNP gene, which then cooperates with the mutated <i>SQSTM1</i> to initiate the exaggerated pattern of bone cell growth characteristic of PDB. We are presently testing this model. We have had difficulty cloning the MVV gene but propose a number of strategies to overcome this problem.					
15. SUBJECT TERMS Paget's disease of bone, Measles virus, osteoclast, osteoblast, disease initiation, latent viral infection, <i>Sequestosome 1</i> , Measles V gene, Measles Nucleocapsid gene					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT Unclassified	18. NUMBER OF PAGES 8	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT Unclassified	b. ABSTRACT Unclassified	c. THIS PAGE Unclassified			19b. TELEPHONE NUMBER (include area code)

Table of Contents

	<u>Page</u>
1. Introduction.....	4
2. Keywords.....	4
3. Overall Project Summary.....	4
4. Key Research Accomplishments.....	7
5. Conclusion.....	7
6. Publications, Abstracts, and Presentations.....	7
7. Inventions, Patents and Licenses.....	7
8. Reportable Outcomes.....	7
9. Other Achievements.....	7
10. References.....	7
11. Appendices.....	8

Introduction

The long-term goal of this project is to understand how Paget's Disease of Bone (PDB) begins. PDB can be inherited in families and several genes have been linked to PDB including *Sequestosome 1 (SQSTM1)*, which has been linked to 40% of familial PDB¹. Measles virus infection has also been linked to PDB in a substantial fraction of cases^{2,3}. One of the key questions in PDB is the nature of the "trigger" for initiation of the disease⁴. As an illustrative example, individuals in a family with familial PDB inherit a germline mutation in the *SQSTM1* gene that predisposes those individuals to PDB. A predisposed individual contracts measles at age 8, which is the only other known factor for PDB. However, the disease does not reveal itself until that individual reaches age 50. What has occurred in the intervening time period that was not present at age 8 is the basis for this proposal. Our model involves the measles virus. The measles virus genome contains a number of genes. Two of these genes, the MVNP protein and the MVV gene appear to act in opposing fashion to control gene expression in an infected cell^{2,5-9}. The MVV gene suppresses gene expression as part of the virus' ability to evade immunological surveillance and promote persistent infection^{5,6}. In contrast, the MVNP gene appears to activate gene expression to promote acute infection¹⁰⁻¹². Our model is that when the measles virus infects a bone cell, the MVV gene creates a latent infection state by suppressing gene expression in the cell. Then, over the years, a chance genetic event in a single bone cell containing the measles virus results in the loss of the MVV gene. At this time, the MVNP gene is unmasked and cooperates with the mutated *SQSTM1* to initiate the exaggerated pattern of bone cell growth characteristic of PDB. To test this model, we proposed two specific aims. In the first aim, we proposed to introduce both the MVV and MVNP genes into bone cells in culture and then use siRNA technology to turn off the MVV gene and observe the change in the growing bone cells to see if it mimics PDB. In the second aim, we proposed to examine affected bone tissue from patients with PDB to see if the affected bone has evidence of mutation of the MVV gene while maintaining the MVNP gene. If our hypothesis is true, then this will dramatically alter our understanding of how PDB initiates. It will also enlighten our understanding of the relationship between measles and PDB.

Keywords

Paget's disease of bone, Measles virus, osteoclast, osteoblast, disease initiation, latent viral infection, *Sequestosome 1*, V gene, Nucleocapsid gene

Overall Project Summary

We have requested a No-Cost Extension to complete the work proposed in this application. We believe that the unexpected difficulties that we encountered during the funding year can be overcome and the work completed with an extension of time. Thus in addition to the description of the work done in the last year, we also propose solutions that we think can overcome the problems.

There were two specific aims to the proposal. The first specific aim was to determine whether loss of expression of Measles virus V (MVV) gene in the presence of Measles virus NP (MVNP) gene leads to a pagetic phenotype. In this aim there were three tasks. The first task was to clone the open reading frame of the MVV gene and the MVNP gene in such a fashion as to allow the two genes to be co-transfected and detected using different fluorescent tags. Our plan was to compare the expression of the MVV gene with the expression of the MVNP gene, which is critical for the onset of Paget's disease. However, the MVV gene is a part of the measles virus

genome in which all three reading frames encode distinct genes (MVP, MVV and MVC) using the same sequence. Our problem has been to clone and express the MVV gene, which contains a non-templated RNA edit. Thus far, we have been unsuccessful.

The problem is shown in Figure 1. The MVP and MVV genes are encoded by a common sequence in the measles virus genome in which both use the ATG start site at nucleotide 1807 and from 1807 to 2498 show a common translated amino acid sequence. The two sequences diverge due to a non-templated insertion of a G nucleotide at nucleotide 2499 in the MVV sequence resulting in a frameshift and altered reading resulting in a unique amino acid sequence for MVP and MVV from that point on. Our attempts at cloning this sequence of the MVV gene from the Measles' genome using a PCR-based strategy in which we assemble the MVV gene from the measles virus genomic RNA using overlapping primer sets have not been successful. As a result, what was expected to be completed in the first six months of the proposal has taken the complete year and still not been successful. We have two proposed solutions to this problem. The first is to adopt a two-step procedure where a truncated MVP gene sequence (approximately nucleotides 1800 - 3000) is amplified by PCR and cloned into a vector and then the non-templated MVV G nucleotide at 2499 is inserted into the cloned MVP gene sequence by a second PCR reaction or restriction reaction. This may reduce the problem of trying to insert a non-templated G directly into the RNA sequence from the total Measles genome since we will have increased the quantity of template we can use. Our second alternative is to isolate the MVV RNA from cells infected with the intact Measles virus and then use that RNA as template to clone the MVV gene. This has the advantage of letting the virus insert the non-templated G base into the RNA sequence. However, this is our second choice because we had been attempting to avoid having intact Measles virus in the laboratory but this may be our only solution to the problem. Once the MVV RNA is isolated, we can use it as a template to reverse transcribe it and clone it into a suitable vector. In either case, we can then transfect the cloned MVV gene together with the MVNP gene. Specific Aim 1 tasks II and III can then be accomplished once the MVV and MVNP genes are co-expressed in the appropriate cells.

Specific Aim 2 was to screen matched normal and pagetic bone samples for evidence of MVV and MVNP and demonstrate that the MVV present in the pagetic samples is mutated in a way that causes inactivation of the MVV gene product. Here we had difficulty with the qRT PCR amplification of the MVV gene. We were not able to detect amplification of the MVV genes in the formalin-fixed paraffin-embedded (FFPE) tissue samples. This is possibly due to our choice of primers, which needed to distinguish MVP from MVV, resulting in a primer sequence location that was not optimal for qRT PCR. Without a cloned MVV gene as a positive control, it is difficult to determine what went wrong. As an alternative approach, we propose to try a ligand-mediated amplification-based assay, which should allow us to distinguish MVP from MVV by the difference in the non-templated inserted G that distinguishes MVP from MVV. We also anticipate that a successful cloning of the MVV gene and subsequent transfection of a control cell line will allow us to troubleshoot the process and succeed in amplifying and detecting the MVV gene in the FFPE tissues. At this point, the amplified gene products can be sequenced to complete our Specific Aim 2 tasks.

Personnel receiving pay for research effort were Marc F. Hansen, Principal Investigator; Michael Mogass, Postdoctoral Fellow; Cindy Alander, Research Assistant.

1807 **ATG**gcagaagagcaggcacgcc**ATG**tcaaaacggactggaatgtatccgggtctcaag
M A E E Q A R H V K N G L E C I R A L K MVP gene
M A E E Q A R H V K N G L E C I R A L K MVV gene
M S K T D W N V S G L S R MVC gene

1867 gccgagcccatcggtcgctggccgtcgaggaagccatggcagcatggtcagaaatatca
A E P I G S L A V E E A M A A W S E I S MVP gene
A E P I G S L A V E E A M A A W S E I S MVV gene
P S P S A R W P S R K P W Q H G Q K Y Q MVC gene

1927 gacaacccaggacaggaccgagccacctgcaaggagaagagcaggcagttcgggtctc
D N P G Q D R A T C K E E K A G S S G L MVP gene
D N P G Q D R A T C K E E K A G S S G L MVV gene
T T Q D R T E P P A R K R R Q A V R V S MVC gene

1987 agcaaacctatgcctctcagcaattggatcaactgaaggcggatgcacctcgcacccgggt
S K P C L S A I G S T E G G A P R I R G MVP gene
S K P C L S A I G S T E G G A P R I R G MVV gene
A N H A S Q Q L D Q L K A V H L A S A V MVC gene

2047 caggatctggagagcagatgacgacgtgaaactttgggaatccctcaagaatctc
Q G S G E S D D D A E T L G I P S R N L MVP gene
Q G S G E S D D D A E T L G I P S R N L MVV gene
R D L E R A M T T L K L W E S P Q E I S MVC gene

2107 caggcatcaagcactgggtacagtgttatcatgtttatgatcacagcggtgaagcggtt
Q A S S T G L Q C Y H V Y D H S G E A V MVP gene
Q A S S T G L Q C Y H V Y D H S G E A V MVV gene
R H Q A L G Y S V I M F M I T A V K R L MVC gene

2167 aagggaatccaagatgctgactctatcatgttcaatcaggccttgatggatgacacc
K G I Q D A D S I M V Q S G L D G D S T MVP gene
K G I Q D A D S I M V Q S G L D G D S T MVV gene
R E S K M L T L S W F N Q A L M V I A P MVC gene

2227 ctctcaggaggagacgatgaatctgaaacacgcatgtggatattggcgaacctgatacc
L S G G D D E S E N S D V D I G E P D T MVP gene
L S G G D D E S E N S D V D I G E P D T MVV gene
S Q E E T M N L K T A M W I L A N L I P MVC gene

2287 gagggatattgctatcactgaccgggatctgctccatctctatgggttcagggtctct
E G Y A I T D R G S A P I S M G F R A S MVP gene
E G Y A I T D R G S A P I S M G F R A S MVV gene
R D M L S L T G D L L P S L W G S G L L MVC gene

2347 gatgttgaactgcagaaggaggtgagatccacgagctcctgagactccaatccagaggc
D V E T A E G G E I H E L L R L Q S R G MVP gene
D V E T A E G G E I H E L L R L Q S R G MVV gene
M L K L Q K E V R S T S S - MVC gene

2407 aacaactttccgaagcttgggaaactctcaatgttcctccgccccgaaccccgtagg
N N F P K L G K T L N V P P P P N P G R MVP gene
N N F P K L G K T L N V P P P P N P G R MVV gene

2467 gccagcgttccgagacaccattaaaaagcgcagacgcgagattagcctcatttgga
A S A S E T P I K K G T D A R L A S F G MVP gene
A S A S E T P I K K G H R R E I S L I W MVV gene

2527 acggagatcgctctttattgacaggtgtgcaaccaatgtgctcgaagtcaccctcg
T E I A S L L T G G A T Q C A R K S P S MVP gene
N G D R V F I D R W C N P M C S K V T L MVV gene

2587 gaaccatcaggccaggtgcacctgtgggaatgtccccgagtggtgagcaatgccgca
E P S G P G A P V G N V P E C V S N A A MVP gene
G T I R A R C T C G E C P R V C E Q C R MVV gene

2647 ctgatacaggagtggacaccgaatctggtaccacaatctccccgagatcccagaataat
L I Q E W T P E S G T T I S P R S Q N N MVP gene
T D T G V D T R I W Y H N L P E I P E - MVV gene

2707 gaagaaggggagactattatgatgatgagctgttctccgatgtccaagacatcaaaaca
E E G G D Y Y D D E L F S D V Q D I K T MVP gene

2767 gccttgccaaaatacagaggataatcagaagataatctctaactagaatcactgctg
A L A K I H E D N Q K I I S K L E S L L MVP gene

2827 ttattgaaggagaggttgagtcaattaagaagcagattaacaggcaaaatcagcata
L L K G E V E S I K K Q I N R Q N I S I MVP gene

2887 tccaccttgaaggacacctctcaagcatcatgatcgccattcctggacttgggaaggat
S T L E G H L S S I M I A I P G L G K D MVP gene

2947 cccaacgacccactgcagatgtcgaactcaatcccgacttgaacccatcataggcaga
P N D P T A D V E L N P D L K P I I G R MVP gene

3007 gattcaggccgagcactggccgaagttctcaagaaacccgctgccagccgacaactccaa
D S G R A L A E V L K K P A A S R Q L Q MVP gene

3067 ggaatgacaaatggacgaccagttccagaggacagctgctgaaggaaattccaactaaag
G M T N G R T S S R G Q L L K E F Q L K MVP gene

3127 ccgactcgggaaaaaggtgagctcagcgtcggtttgtccctgacacccggcgtgtatca
P I G K K V S S A V G F V P D T G P V S MVP gene

3187 cgcagtgtaatccgctcattataaaatccagtcggctagaagaggatcgaagcgttac
R S V I R S I I K S S R L E E D R K R Y MVP gene

3247 ctgatgactctccttgatgatatacaaggagccaacgatcttgccaagttccaccagatg
L M T L L D D I K G A N D L A K F H Q M MVP gene

3307 ctgatgaagataataatgaagtag
L M K I I M K - MVP gene

Figure 1. Sequence comparison of the open reading frames of Measles virus P/V/C genes. The shared ATG start of the MVP/MVV genes is shown in red. The shared amino acid sequence of MVP/MVV is shown in black. The unique amino acid sequence of MVV, caused by a non-templated G insertion into the sequence at nucleotide 2499 (site of insertion is boxed in red, inserted base not shown), is shown in red. The start ATG for MVC and the unique amino acid sequence are shown in blue. Sequence from GenBank NC_001498.1.

Key Research Accomplishments

Nothing to report

Conclusion

This work has the potential to significantly alter our understanding of Paget's disease. For the past 37 years, the role of Measles virus in Paget's disease has been a highly controversial question. Our model has the potential to address this question directly and to provide a model for the role of Measles virus and how Paget's disease initiates.

As noted, we have requested a no-cost extension of the grant to complete the work. We have identified several alternative strategies, which are outlined in the Overall Project Summary, to overcome the problems that we have encountered in the experimental design of both aims. With the additional time requested in the no-cost extension, we will be able to complete the objectives of the grant.

Publications, Abstracts and Presentations

Nothing to report

Inventions, Patents and Licenses

Nothing to report

Reportable Outcomes

Nothing to report

Other Achievements

Nothing to report

References

1. Laurin N, Brown JP, Morissette J, Raymond V. Recurrent mutation of the gene encoding sequestosome 1 (SQSTM1/p62) in Paget disease of bone. *Am J Hum Genet* 2002;70:1582-1588.
2. Kurihara N, Hiruma Y, Yamana K, et al. Contributions of the measles virus nucleocapsid gene and the SQSTM1/p62(P392L) mutation to Paget's disease. *Cell Metab* 2011;13:23-34.
3. Roodman GD. Insights into the pathogenesis of Paget's disease. *Ann N Y Acad Sci* 2010;1192:176-180.
4. Bolland MJ, Tong PC, Naot D, et al. Delayed development of Paget's disease in offspring inheriting SQSTM1 mutations. *J Bone Miner Res* 2007;22:411-415.
5. Schuhmann KM, Pfaller CK, Conzelmann KK. The measles virus V protein binds to p65 (RelA) to suppress NF-kappaB activity. *J Virol* 2011;85:3162-3171.
6. Indoh T, Yokota S, Okabayashi T, Yokosawa N, Fujii N. Suppression of NF-kappaB and AP-1 activation in monocytic cells persistently infected with measles virus. *Virology* 2007;361:294-303.
7. Kurihara N, Reddy SV, Menaa C, Anderson D, Roodman GD. Osteoclasts expressing the measles virus nucleocapsid gene display a pagetic phenotype. *J Clin Invest* 2000;105:607-614.

8. Kurihara N, Zhou H, Reddy SV, et al. Expression of measles virus nucleocapsid protein in osteoclasts induces Paget's disease-like bone lesions in mice. *J Bone Miner Res* 2006;21:446-455.
9. Helin E, Vainionpaa R, Hyypia T, Julkunen I, Matikainen S. Measles virus activates NF-kappa B and STAT transcription factors and production of IFN-alpha/beta and IL-6 in the human lung epithelial cell line A549. *Virology* 2001;290:1-10.
10. Noe KH, Cenciarelli C, Moyer SA, Rota PA, Shin ML. Requirements for measles virus induction of RANTES chemokine in human astrocytoma-derived U373 cells. *J Virol* 1999;73:3117-3124.
11. tenOever BR, Servant MJ, Grandvaux N, Lin R, Hiscott J. Recognition of the measles virus nucleocapsid as a mechanism of IRF-3 activation. *J Virol* 2002;76:3659-3669.
12. Takayama I, Sato H, Watanabe A, et al. The nucleocapsid protein of measles virus blocks host interferon response. *Virology* 2012;424:45-55.

Appendices

Nothing to report